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Alterations in G1 Phase Cell Cycle Regulation during the Development of Benzo[a]pyrene-induced Epithelial Dysplasia in the Murine Tongue

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Abstract: The environmental contaminant benzo[a]pyrene (B[a]P) has been regarded as one of the pathogens of oral premalignant and malignant lesions. To elucidate the pathogenesis of oral premalignancies, B[a]P-induced dysplasia of the murine tongue was investigated for G1-associated cell cycle regulation. B[a]P solution was applied orally up to six weeks to induce epithelial dysplasia of the tongue. BrdU incorporation and the expression of p21, cyclin D1, and CDK4 were examined by immunohistochemistry and Western blotting. Rb phosphorylation and E2F-Rb binding were examined by immunoprecipitation and Western blotting. B[a]P treatment resulted in dysplastic changes and active DNA synthesis in the tongue epithelia. Immunohistochemical analyses showed p21 up-regulation and cyclin D1/CDK4 overexpression in B[a]P-induced dysplasia. Rb hyperphosphorylation and E2F release were caused by B[a]P treatment. Thus, dysregulation of G1-phase regulation is likely to be an important event in the development of oral epithelial dysplasia in mice.

Key words: oral epithelial dysplasia, benzo[a]pyrene, cell cycle, mouse

Introduction

The *p53* tumor suppressor gene and its downstream factors act as key regulators of inhibition of the G1 to S phase transition. G1-S progression in the mammalian cell cycle is regulated by phosphorylation of Retinoblastoma (Rb) family proteins and subsequent release of E2F transcription factor[1,2]. Cyclin-dependent kinases (CDKs)-2, -4, and -6 are active on association with their catalytic partner, cyclin D or E, and are responsible for hyperphosphorylation of Rb[1,2]. The kinase activity of CDKs is negatively regulated by a series of CDK inhibitors (CKIs): the CIP/KIP family (p21, p27, and p57) and INK4 family (p16, p15, p18, and p19)[3]. *p21*, the first CKI to be identified and characterized, was initially reported as the downstream effector of *p53*, and its overexpression results in cell cycle arrest[4].

Abnormalities in G1 phase cell cycle regulation have been implicated in the pathogenesis of several types of human malignancies. *p21* has been intensively studied for its contribution to tumor suppression. Van Oijen *et al.*[5] reported a strong negative correlation between the expression of *p21* and the proliferative potential of head

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and neck carcinoma cells. Amplification, overexpression, and rearrangement of cyclin D1 gene are frequently observed in various malignant tumors including head and neck squamous cell carcinomas[6]. Also, studies on rodent models of experimental tumors revealed that cyclin D1 overexpression occurs in the two-stage chemical carcinogenesis of murine skin[7]. Transgenic overexpression of wild-type cyclin D1 gene resulted in abnormal proliferative responses of epidermal keratinocytes in mice[8]. Another report showed that a cyclin D1 conditional transgene with a keratin-5 promoter and skin-specific overexpression enhanced the susceptibility of dimethylbenz[a]anthracene (DMBA)-induced skin tumorigenesis[9]. In contrast, mice deficient for cyclin D1 gene displayed a reduced incidence of v-Ha-ras-dependent, DMBA-induced skin papilloma[10]. These previous reports suggest that disruption of G1 phase regulation leads to unregulated growth and tumor promotion. However, less is known about the changes in cell cycle regulation during the formation of epithelial dysplasia of the oral mucosa.

Tobacco-associated polycyclic aromatic hydrocarbon benzo[a]pyrene (B[a]P) is an indirect carcinogen ubiquitous in the environment[11]. Its electroreactive metabolites act as strong mutagens in various organs by forming DNA adducts[12–15]. Because this compound has been implicated in the etiology of malignant tumor formation in the respiratory and digestive tracts, intensive investigations have been done on B[a]P-induced experimental carcinogenesis in the trachea[16] and stomach[17]. Repeated oral application of B[a]P solutions efficiently produces tumors in the buccal pouch and tongue of rodents[18, 19]. Park *et al.* reported that chronic exposure to B[a]P resulted in malignant transformation of HPV16-immortalized human oral keratinocytes *in vitro*[20]. Although many studies have been directed at the malignant transformation of B[a]P-exposed tissue and cultured cells, the critical events that occur during the formation of oral epithelial dysplasia have not been examined yet. For more sophisticated diagnosis and treatment of oral leukoplakia and other precancerous lesions, it is necessary to understand the pathogenic mechanisms of the dysplasia.

In the current study, we focused on the possible alterations in the regulation of G1 to S phase cell cycle progression and analyzed the changes in the expression of G1-related regulators on a mouse model of B[a]P-induced oral epithelial dysplasia.

Materials and methods

1. Animals and carcinogen treatment

C57BL6J mice were purchased from Sankyo Laboratory Service (Tokyo, Japan). Animals were bred in specific pathogen-free conditions and used for experiments between 6 and 12 weeks of age. Mice were divided into two groups (five mice for each treatment period) and control (five mice). Twenty μl of tricaprylin (for control) or an equal volume of 1.0% (w/v) B[a]P (Sigma, St Louis, MO) in tricaprylin (for experimental) was administered *per os* daily for one to six weeks. Mice were then euthanized by CO_2 inhalation and the tongue tissue was immediately resected. For immunoprecipitation and Western blotting, the resected tissue was snap-frozen in liquid nitrogen and stored at −80℃ until use. For histology, the resected tissue was immediately fixed in 10% neutral-buffered formalin and embedded in paraffin.

2. Antibodies (Abs)

Rabbit anti-mouse p16, p27, Rb, cyclin E, CDK2, and CDK4 polyclonal Abs (immunoglobulin (Ig) G), mouse anti-mouse E2F-1 (IgG2a), cyclin D1 (IgG1), and p21 (IgG1) monoclonal Abs were purchased from Santa Cruz Biochemistry (San Diego, CA). Mouse monoclonal anti-mouse p21 Ab (IgG1) was purchased from Pharmingen (San Diego, CA). Biotinylated anti-BrdU Ab was purchased from Medical and Biological Laboratories (Nagoya, Japan).

3. 5-bromodeoxyuridine (BrdU) incorporation

Mice were injected *i.p.* with BrdU (100 mg/kg body weight in 200 μl PBS) 16 h before euthanization. Serial sections of tongue tissue were deparaffinized, and incubated in 2 M HCl for 30 minutes at 37℃. Endogenous peroxidase was reduced by preincubating with 0.3% H_2O_2. The sections were then trypsinized and stained with biotinylated anti-BrdU Ab followed by peroxidase-conjugated streptavidin. Immune complexes were detected with diaminobenzidine.

4. Immunohistochemistry

Serial sections of 4-μm thickness were prepared and

deparaffinized with xylene and ethanol. Immunohistochemical staining was performed with a Histofine® SAB-PO kit (Nichirei, Tokyo, Japan) according to the manufacturer's instructions. Briefly, sections were quenched with 0.3% H_2O_2 to reduce intrinsic peroxidase activity and blocked with 10% normal goat serum. Sections were incubated for 2 h at room temperature in a humidified chamber with primary Abs or their isotype-matched control Ig. After incubating with biotin-conjugated second anti-IgG for 30 min at room temperature, peroxidase-conjugated streptavidin solution was applied to all sections for 30 min. Immune complexes were detected with DAB.

5. Immunoprecipitation

Cell lysate was precleared with normal mouse serum and Protein G Sepharose beads (Amersham Pharmacia Biotech, Uppsala, Sweden). Precleared lysate was incubated with Protein G Sepharose beads conjugated with primary Ab. After 2-h incubation at 4℃ under constant agitation, the beads were washed in lysis buffer and boiled for 10 min in 2x SDS loading buffer (0.1 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.1% mercaptoethanol, 0.004% bromophenol blue) to elute the conjugated protein. The supernatant was separated by SDS-PAGE and subjected to subsequent Western immunoblotting.

6. Western blotting

The frozen samples were homogenized and protein was solubilized in 0.25% IGEPAL lysis buffer (20 mM HEPES pH 7.5, 350 mM NaCl, 25% glycerol, 0.25% IGEPAL CA-630, 1 mM sodium *o*-vanadate) supplemented with a Complete Mini™ Protease Inhibitor Tablet (Roche Diagnostics, Mannheim, Germany). The cell lysate was clarified by centrifugation, and protein was separated by SDS-PAGE and transferred to a Hybond™ PVDF membrane (Amersham Pharmacia). The membrane was blocked with skim milk and reacted with primary Ab for 90 min at room temperature. The membrane was then washed and reacted with HRP-conjugated secondary Ab. Protein bands were visualized with an ECL™ Western blot detection system (Amersham Pharmacia).

Results

1. Histological changes in murine tongue epithelia induced by B[a]P treatment

Observation of the excised tongue under dissection microscope showed that B[a]P did not induce tumor formation in the tongue within six weeks. Fig. 1 shows hematoxylin-eosin (HE)-stained sections of the tongue tissue from normal (Fig. 1a) and B[a]P-treated (Fig. 1b-d) mice. One-week treatment with B[a]P was insufficient to induce any marked changes in the tongue epithelium (Fig. 1b). However, mild to moderate dysplastic changes of the tongue epithelium occurred after three-week treatment (Fig. 1c). Morphological alterations such as loss of polarity of the basal cells, basaloid appearance of the suprabasal cells, increased nucleus/cytoplasm ratio and enlarged nucleoli of the prickle cells, cytoplasmic hyperchromatism, and thickening of the keratinized layers were seen in epithelia from mice treated with B[a]P. These features were more remarkably observed in the tissue from the six-week-treated mice (Fig. 1d) An increase in mitotic figures or nonphysiologic mitoses was not seen at any time (Fig. 1b-d).

2. BrdU incorporation in tongue epithelial cells treated with B[a]P

We next carried out *in vivo* BrdU incorporation experiments to evaluate the DNA synthesizing activity of B[a]P-exposed cells. Basal cells of the untreated normal epithelium were weakly positive for BrdU staining; only a very small number of suprabasal cells were BrdU-positive (Fig. 2a). B[a]P treatment remarkably increased BrdU incorporation in basal and suprabasal cells within one week (Fig. 2b). Strong positive staining for BrdU was noted in the majority of prickle cells in the epithelia treated with B[a]P for three (Fig. 2c) and six weeks (Fig. 2d).

3. Changes in the expression of *p21* in tongue epithelium induced by B[a]P

We next examined whether the genotoxic stresses due to B[a]P treatment activate the p21-dependent cell cycle regulation cascade. A small number of cells in the normal tongue were found to be weakly positive for p21 (Fig. 3a). After a six-week treatment with B[a]P, this CKI was markedly induced in the epithelia, especially

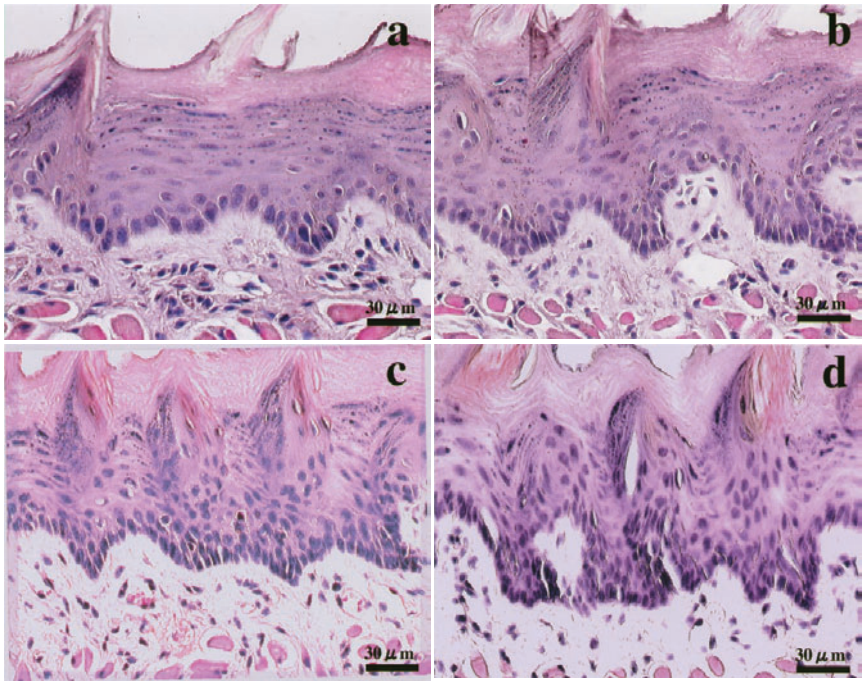

Fig. 1 Epithelial dysplasia of murine tongue mucosa induced by B[a]P. Mice were treated by the oral application of tricaprylin (a) or a 0.1% w/v tricaprylin solution of B[a]P for one (b), three (c), or six (d) weeks. Mice were euthanized and sections of the tongue were stained with HE. (Bars, 30 μm)

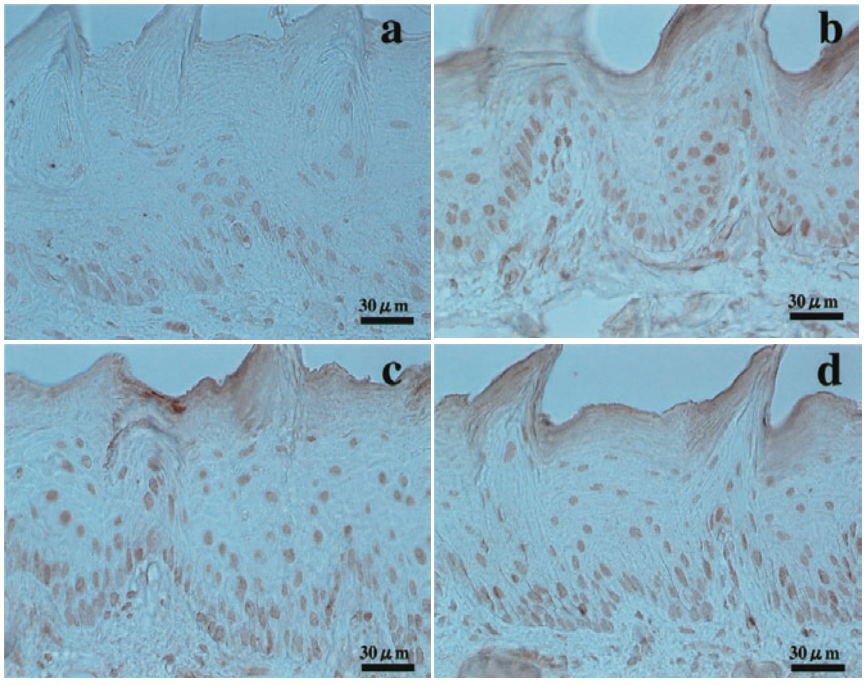

Fig. 2 Incorporation of BrdU in normal and B[a]P-treated tongue epithelia. Mice were treated by the oral application of tricaprylin (a) or a 0.1% w/v tricaprylin solution of B[a]P for one (b), three (c), or six (d) weeks, and BrdU was injected *i.p.* 16 h before euthanization. BrdU incorporation in all the layers of the epithelium of the tongue was examined by anti-BrdU immunohistochemical staining. (Bars, 30 μm)

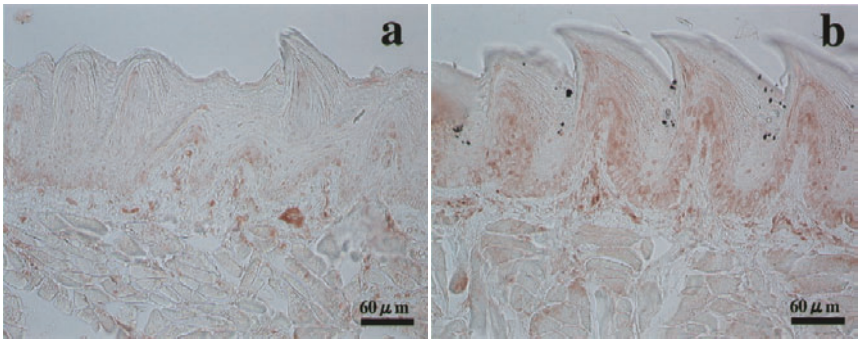

Fig. 3 Distribution of p21 protein in B[a]P-treated tongue epithelia.
Mice were treated with tricaprylin (a) or a tricaprylin solution of B[a]P for six weeks (b). p21 protein in the tongue epithelium was detected by immunohistochemistry. (Bars, 60 μm)

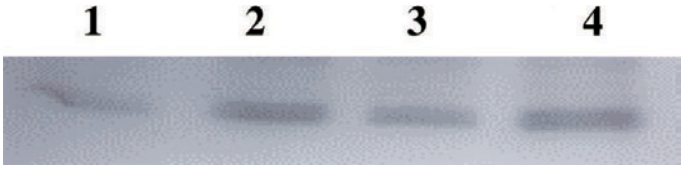

Fig. 4 Western blot analysis of p21 expression in B[a]P-treated tongue tissue.
Mice were treated by the oral application of tricaprylin or tricaprylin solution of B[a]P for one to six weeks. Expression of p21 was analyzed by Western blotting as described in Materials and Methods. Lane 1: tricaprylin-treated control. Lanes 2, 3, and 4: treated with B[a]P for one, three, and six weeks, respectively. Representative data from three individual experiments are shown.

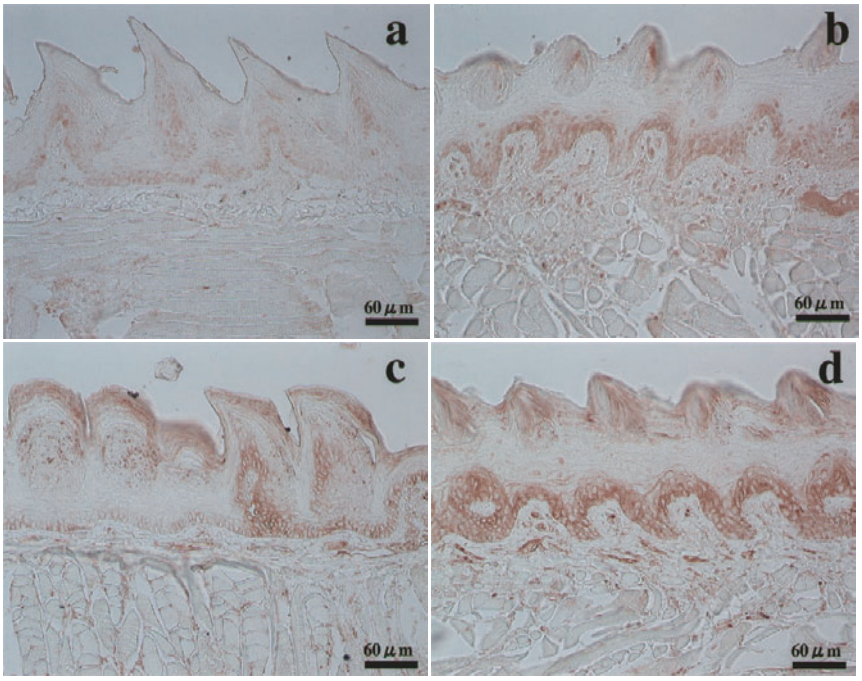

Fig. 5 Expression of cyclin D and CDK4 in B[a]P-treated tongue tissue.
Mice were treated with tricaprylin (a, c) or a tricaprylin solution of B[a]P for six weeks (b, d). Cyclin D and CDK4 were detected by immunohistochemistry. Representative data from three individual experiments are shown. (Bars, 60 μm)

in the basal cell layer (Fig. 3b). Similar results were obtained from Western blot analysis (Fig. 4). Constitutive p21 expression was also detected in the immunoblot assay. B[a]P treatment up-regulated the expression of p21 in the tongue tissue. These results suggested that B[a]P might induce the p21-dependent inhibition of cell cycle progression. Expression of CKIs p16, p27, and p57 was also examined by immunohistochemistry and Western immunoblotting, but no detectable changes were observed (data not shown).

4. Changes in the expression of cyclin D1 and CDK4 induced by B[a]P

Immunohistochemical staining showed that cyclin D1 immunoreactivity was almost undetectable in untreated normal epithelium (Fig. 5a). Only a small number of basal cells were weakly positive for cyclin D1. On the other hand, positive cyclin D1 staining was observed in the basal cell layer of six-week-treated epithelium (Fig. 5b). Cyclin D1 was also detected in some of the suprabasal cells, but the prickle cells were negative for this protein. The expression of this protein was time-dependently augmented by the treatment (data not shown). Similar results were obtained from the immunohistochemistry for CDK4 (Fig. 5c and d).

Western immunoblot assay was also performed to examine the expression of cyclin E/CDK2, but marked changes were not induced by B[a]P treatment (data not shown).

5. Hyperphosphorylation of Rb and release of E2F in B[a]P-treated tongue epithelium

For the next set of experiments, Western blot analysis was performed to examine the phosphorylation status of Rb. Using a primary antibody which recognizes both hyperphosphorylated (ppRb) and hypophosphorylated (pRb) forms of the protein, Rb appears as two distinct bands: higher molecular weight ppRb and lower molecular weight pRb. pRb was dominant in the control tissue (Fig. 6; lane 1), but ppRb was remarkably increased by B[a]P in a time-dependent manner. These data suggested that the phosphorylation level of Rb was elevated in B[a]P-induced epithelial dysplasia.

Next, the association of Rb and E2F in the untreated and B[a]P-treated tissues was examined by immunoprecipitation and subsequent Western immunoblotting (Fig. 7). A distinct Rb band was observed in the sample from untreated tissue (Fig. 7; lane 1). Exposure to B[a]P resulted in a marked time-dependent decrease in the Rb band intensity (Fig. 7; lanes 2-4), suggesting that E2F release from Rb was notably enhanced in B[a]P-induced epithelial dysplasia through Rb hyperphosphorylation.

Discussion

B[a]P is a widespread and persistent pollutant contained in tobacco smoke and some types of food[11]. This compound itself is non-genotoxic, but its metabolite B[a]P diol epoxide (BPDE) acts as a strong mutagen[12–15]. These metabolically-activated mutagens react with DNA to form BPDEs-DNA adducts, resulting in DNA mis-replication at the affected sites[21]. When the DNA lesions are formed at the critical sites for tumor suppression or oncogenesis and fail to be properly and promptly repaired, the cells are directed to the premalignant condition, and then to malignant transformation. In the current study, changes in cell cycle regulation during the development of epithelial dysplasia were investigated using a murine model of B[a]P-induced oral epithelial dysplasia. B[a]P dissolved in tricaprylin was orally applied, and mild to moderate dysplastic changes of the epithelia were successfully induced within six weeks after the start of the B[a]P treatment. Findings from histological analyses of B[a]P-treated tongue epithelium (Fig. 1) suggested that the differentiation/proliferation properties of the epithelial cells were altered by chronic exposure to this DNA damaging agent. Increases in mitotic figures or development of carcinoma *in situ* were not seen in these experiments. Oral mucosal tissue from all B[a]P-treated animals appeared macroscopically normal. Some of the mice in the experimental group died probably due to drug-induced liver dysfunction before oral tumors could develop. To observe cancer development in the murine system, an extended treatment period with lower carcinogen concentration or multistep induction of carcinogenesis with other mutagens might be required. We also used another genotoxic chemical, 4-nitroquinoline-*n*-oxide (4-NQO), which has been widely used for rat models of oral carcinogenesis, but oral mucosa of C57BL6 mice appeared to be more sensitive to B[a]P than to 4-NQO in terms of the susceptibility of epithelial dysplasia

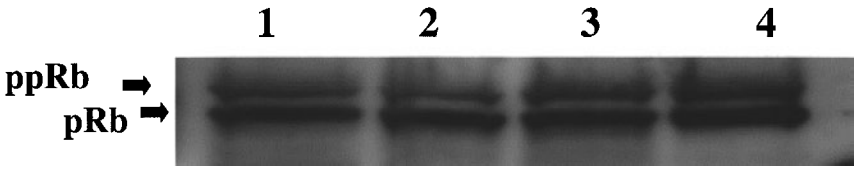

Fig. 6 Phosphorylation status of Rb protein in B[a]P-treated murine tongue. Mice were treated with tricaprylin or B[a]P in tricaprylin for one to six weeks. Resected tongue was homogenized, and protein was solubilized by lysis buffer. Expression of Rb was analyzed by Western blotting. Rb was detected as two distinct bands; the hyperphosphorylated form (ppRb) with a slower migration rate and the hypophosphorylated form with higher migration rate (pRb). Lane 1: tricaprylin-treated control. Lanes 2, 3, and 4: treated with B[a]P for one, three, and six weeks, respectively. Representative data from three individual experiments are shown.

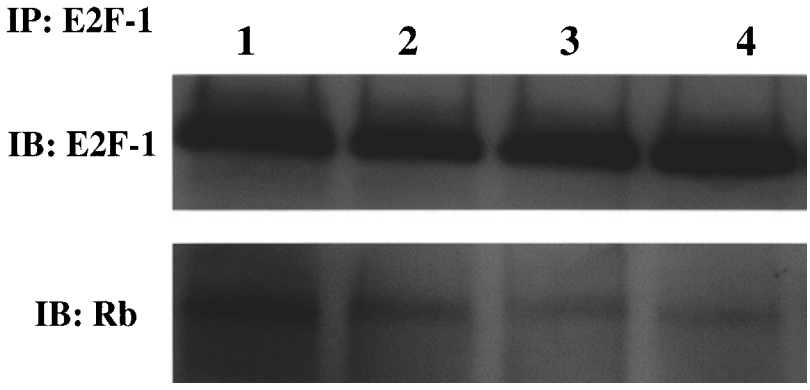

Fig. 7 Effect of B[a]P on the binding of Rb and E2F in murine tongue. Mice were treated by oral application of tricaprylin or a tricaprylin solution of B[a]P for one to six weeks. Lysate of the resected tongue tissue was immunoprecipitated (IP) with Protein G Sepharose and anti-E2F monoclonal antibody. The E2F immunoprecipitate was separated by SDS-PAGE and immunoblotted (IB) with anti-E2F (upper panel) and Rb (lower panel) monoclonal antibodies. Lane 1: tricaprylin-treated control. Lanes 2, 3, and 4: treated with B[a]P for one, three, and six weeks, respectively. Representative data from three individual experiments are shown.

(data not shown).

The data from immunohistochemical staining and immunoblots for p21 demonstrated that B[a]P exposure augmented the expression of this protein, especially in the basal cell layers. Once genomic DNA is damaged, induced and activated p21 disturbs the entry into S phase, providing a chance to repair DNA lesions. The augmented expression of p21 noted in our data could be regarded as a defensive reaction against B[a]P toxicity. Expression of its upstream regulator p53 was not affected by B[a]P (data not shown), corresponding with previous reports that B[a]P activates the p53-independent cell cycle-arresting cascade *in vitro*[21,22]. B[a]P might activate the p21-dependent/p53-independent pathway of growth suppression during the G1 phase.

In spite of p21 induction, enhanced incorporation of BrdU indicated up-regulated DNA synthesizing activity in B[a]P-treated epithelial cells. Some of the suprabasal cells turned BrdU-positive after B[a]P exposure, suggesting that the cells sustained their growth activity instead of going to the physiological differentiation. p21 was up-regulated by the mitogenic stimuli as a physiological negative-feedback reaction to block nonphysiological acceleration of the cell cycle. We speculate that the B[a]P-induced S-phase promoting forces overcame the blockade by p21. Overproduced p21 protein might be consumed by binding the overproduced cyclin D1/CDKs complexes. Inhibition of cyclin E/CDK2 by p21 might be thus unsuccessful, resulting in the S-phase entry of the cells.

Expression of cyclin D1 and CDK4, the targets of p21, was examined to investigate whether these factors are induced to overcome the p21 blockade, because cyclin D up-regulation has been reported to be associated with malignancies[6–8]. Immunohistochemical staining showed that cyclin D1 and CDK4 were markedly up-regulated in response to B[a]P treatment at the sites where p21 overexpression was observed. These data on cyclin D1 correspond to previous reports on the precancerous lesions of human oral and laryngeal mucosa[23–25]. We have no direct evidence that the cyclin D1/CDK4 complex is enzymatically activated as well as overexpressed. A kinase assay of purified protein is needed to confirm this. However, the cyclin D1/CDK4 substrate Rb was found to be time-dependently hyperphosphorylated (Fig. 6) and release of E2F transcription factor from Rb occurred after B[a]P treatment (Fig. 7), suggesting that the p21-dependent pathway of cell cycle arrest did not properly work to block G1-S progression.

Our results indicated that BrdU-positive cells were distributed mainly in the suprabasal and prickle cell layers (Fig. 2), not in the basal layers where cyclin D1/CDK4 was overexpressed (Fig. 5). The cyclin D1/CDK4 complex works in the early stage of the G1 phase for the progression to the late G1 phase. In the late G1, cyclin E/CDK2 promotes S-phase entry instead of cyclin D/CDK4. Our preliminary data showed that B[a]P treatment up-regulated cyclin E in the suprabasal and prickle cells (data not shown). We speculate that B[a]P accelerates cell cycle progression of the basal cells through an overexpression of the early G1 proteins, resulting in the increase and accumulation of the S-phase cells in the suprabasal and prickle cell layers.

It is likely that B[a]P-exposed epithelial cells are escaping from a physiologically-stable condition and entering the actively proliferating stages. In *in vitro* cultured cells, B[a]P transiently induces cell cycle arrest. Guo *et al.*[21] demonstrated that the cell cycle of Swiss 3T3 cells was first arrested by B[a]P and mitogenic stimuli, and then recovered from arrest and progressed to the G2/M phases. They also noted that the quantified level of DNA damage did not decline during recovery from the arrest and re-entry into cell cycle progression, and mentioned the existence of a cell cycle checkpoint adaptation mechanism. Additionally, Khan *et al.*[26] reported that S phase re-entry occurs when G1-arrested human MCF-7 cells are treated with the B[a]P metabolite BPDE. We did not perform any detailed investigation to examine the very early events induced by B[a]P treatment. However, it is speculated that there is a similar checkpoint adaptation during the development of experimental epithelial dysplasia.

Overall, our current study demonstrates an association between dysplastic changes of the oral mucosal epithelia and alterations in G1 cell cycle control in the mouse. The data suggest that continuous carcinogen exposure leads cells to the active proliferation stages *in vivo*. We failed to detect changes in the gene expression of some cell cycle regulatory factors including CKIs other than p21. This is probably because such changes are too small to detect or occur mainly in very restricted numbers of epithelial cells. More sophisticated experimental techniques such as laser micro-dissection will help to solve these problems. The checkpoint adaptation mechanism should be elucidated in future studies for the further understanding of oral epithelial carcinogenesis.

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